Pooled samples and eDNA-based detection can facilitate the "clean trade" of aquatic animals

Jesse L. Brunner[1,*]

[1]Washington State University, School of Biological Sciences, Pullman, WA, 99164 USA
[*]jesse.brunner@wsu.edu

ABSTRACT

The regional and international trade of live animals facilitates the movement, spillover, and emergence of zoonotic and epizootic pathogens around the world. Detecting pathogens in trade is critical for preventing their continued movement and introduction, but, screening a sufficient fraction to ensure even moderately rare infections are detected is simply infeasible for most taxa and settings because of the vast numbers of animals involved. Hundreds of millions of live animals are imported into the U.S.A. every year, for instance. Batch processing pools of individual samples or using environmental DNA (eDNA)—the genetic material shed into an organism's environment—collected from whole consignments of animals may substantially reduce the time and cost associated with pathogen surveillance. Both approaches, however, lack a framework for determine sampling requirements and interpreting results in this context. Here I present formulae with which to make inference from pooled and eDNA samples and discuss key assumptions and considerations for their use with a focus on detecting *Batrachochytrium salamandrivorans*, an emerging pathogen that threatens global salamander diversity. These formulae illustrate how eDNA-based detection, especially, can substantially reduce sample sizes and help bring clean trade into reach for a greater number of taxa, places, and contexts.

Introduction

The regional and international trade in live animals facilitates the inadvertent translocation, spillover, and emergence of zoonotic and epizootic pathogens around the world[1–7]. From the spread of agricultural pathogens (e.g., African swine fever virus, foot and mouth disease virus, or rinderpest virus) to emerging zoonoses (e.g., MERS-CoV, Avian influenza virus), "pathogen pollution"[1,2] can have dramatic socioeconomic impacts and threaten human health[5,8–11]. It is also playing an increasingly important role in wildlife conservation[1,2,9,12,13]. Once established, pathogens are usually difficult if not impossible to eradicate or even control[14,15]. Thus, detecting pathogens in trade in order to prevent their introduction and establishment is a key goal[6,9,16–18].

The largest challenge for routine surveillance of the live animal trade is its magnitude. The pathways of trade are diverse and complex[6,10] so only portions are well characterized, but two estimates are sobering and illustrative. First, over a billion ornamental fish are traded internationally every year[14]. Second, the USA alone imports an average of 225 million individuals in addition to 1.8×10^6 kg of live animals per year, the "vast majority" of which are part of the aquatic and pet industry[6]. While there are national or international regulations governing the trade of certain taxa (e.g., those of economic importance, threats to human health, threatened and endangered species), for most there is little on-the-ground scrutiny and little or no surveillance for disease. Indeed, detection infections with with standard, individual-based sampling—that is, collecting a swab, tissue, or blood sample from each individual for subsequent diagnostic tests (e.g., PCR, ELISA)—requires such large sample sizes as to make routine surveillance infeasible. Even under a best-case scenario (e.g., using a diagnostically perfect test to detect a pathogen occurring at 10% prevalence) one would still need to screen 25 individuals in a consignment of 100 animals; multiplied by the myriad consignments (e.g., $> 10^5$ per year into the USA alone[6]) and the number becomes daunting. More often we are interested in detecting rare infections with imperfect diagnostic tests (i.e., false negatives and false positives occur at some non-trivial rate) and the sample sizes increase concomitantly. While routine surveillance may be possible in certain fractions of the live animal trade that are relatively small or economically important, it is simply out of reach for most taxa and many stakeholders (i.e., nations, importers, etc.).

Two approaches have been suggested to reduce the numbers of samples to more manageable levels. First, individual samples can be pooled and processed as a group[19–21], reducing the level of sample processing (e.g., DNA extractions) and screening (e.g., PCR reactions, ELISAs) several-fold. Second, one might screen environmental DNA (eDNA)—the genetic material shed into an organism's environment—for the pathogen or parasite of interest[22]. The eDNA approach seems especially relevant to aquatic and semi-aquatic taxa, which make up a large portion of the wildlife trade[6], as eDNA can be simply filtered from water housing animals; they need not even be handled. (Semi-aquatic species or life stages are often shipped in moist sphagnum moss or paper, but can then be placed in water to collect eDNA during a quarantine period[23]). However, while both approaches

have the potential to reduce sample sizes, we lack the formal framework for determine sampling requirements and interpreting results. In this paper I therefore develop the theoretical frameworks within which to make inference from pooled samples and eDNA, discuss the key assumptions and considerations in their application, and illustrate how eDNA may be especially useful for detecting infections in the live animal trade. I present these results in the context of emerging fungal pathogens that threaten amphibian diversity, for which trade appears to play a key role.

Chytrid fungi as an exemplar

The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), is the most devastating emerging pathogen on record, responsible for declines in over 500 species, including 90 that are likely extinct[24]. It owes its global distribution in large measure to the international trade of African clawed frogs (*Xenopus laevis*) used for pregnancy tests and research[25], American bullfrogs (*Lithobates catesbeianus*) sold for food[26,27], and myriad species involved in the pet trade[28–32]. More recently, the international pet trade aided the emergence of a novel chytrid fungus, *B. salamandrivorans* (Bsal), that is particularly lethal to salamanders[33–36].

Bsal was introduced into Northern Europe via the pet trade from Southeast Asia[35,37–39]. While it has not yet been detected in the wild in much of Europe[40–43], it is already prevalent in and appears to have spread among private collections in Europe[44–46]. In North America, a hot-spot of salamander diversity, Bsal is apparently absent from both wild and captive amphibians[42,47–49]. However, the risk and potentially devastating consequences of its introduction via trade[50–52] led to prohibitions on the importation of 201 species of salamanders into the U.S.A.[53] and all salamanders in Canada[54].

While laudable, such bans are necessarily crude. These restrictions were necessarily based on experiments with small samples sizes of few species, extended to others by taxonomic affiliation[35]. Moreover, it was quickly out of date since several other taxa, including frogs, are now known to carry Bsal[36,38]. Such bans may also promote black-market trade, which already occurs on a global scale similar to the black-market trade of narcotics[10]. In response to these challenges, as well as the economic interests of the pet trade, there have been calls for a "clean trade" of amphibians[55–57]. Indeed, the European Union recently mandated a program requiring screening, using a quantitative real-time PCR (qPCR) assay of skin swabs[58], or prophylactically treating all consignments of live salamanders into and between member nations of the EU[59].

Again, the scale of the international trade of live amphibians makes routine screening a formidable challenge. While the number of consignments of amphibians into or between EU member nations is not available in the literature, an estimated 3–5 million live amphibians are imported per year into the USA, alone[27,60] and 130,000 annually into the U.K.[61]. Since Bsal is thought to occur at low prevalence in traded animals[38], the EU regulations require screening all or a substantial fraction of individuals in a consignment to ensure a reasonable chance of detection (Fig. 1). Even rather conservative assumptions about the volume of trade imply screening an enormous number of samples (e.g., 1000 consignments of 200 salamanders each would require testing some 98,000 swabs per year). This scale of sampling is likely to place clean trade beyond the reach of many nations. The question in this paper is: can pooling swab samples or using eDNA place routine surveillance in reach?

Results

The formulae presented below can account for false positives (i.e., less than perfect specificity, Sp). It will be important to consider the sources and likelihood of false positives (e.g., from carry over of target DNA from the water rather than infected hosts vs. from contamination in a laboratory) as well as the consequences (e.g., slowing shipments, economic costs, loss of trust[62,63]). In the case of Bsal detection, I would hope that any positive test would be investigated further with additional diagnostic tests. I therefore set aside the issue of specificity in the following discussion and focus on sensitivity.

Pooling individual samples

First proposed by Dorfman[19] in 1943 in the context of screening U.S. service members for syphilis, pooling samples to reduce the number of tests required to detect rare infections is common in numerous contexts[20], although less frequently and consistently in aquatic animals[64]. Only recently, however, has a probability formula been developed for pooled samples that might apply to the problem of detecting infections in consignments or captive populations, i.e., finite populations sampled without replacement[65]. I extend the "pooled hypergeometric" developed by Theobald and Davie[65], to account for imperfect diagnostic tests (equation (3)). The results are largely intuitive: when n individual-level samples are divided into $m = n/k$ pools of size k, the number of pools that need be screened is reduced up to k-fold, assuming low prevalence of infection and high diagnostic sensitivity[64,65]. A key assumption of this formula is that diagnostic sensitivity is not affected by pooling. This implies on the one hand that the combined analyte (e.g., target DNA) from multiple infected individuals does not increase diagnostic sensitivity. The probability of detecting a single infection in a pool of samples (i.e., swabs) is at most the diagnostic sensitivity of individual swabs. On the other hand, the formula assumes that the analyte is not overwhelmed or inhibited by non-target materials (e.g., PCR inhibitors in skin secretions). This later assumption is unlikely to hold with very large pools of

samples (see Laurin et al.[64] for a review), but may be reasonable for small pools. It is an important assumption to test under realistic conditions, but one could simply use lower values of diagnostic sensitivity (*Se* in equation (3)) to account for target swamping or dilution.

In the case of Bsal, Sabino-Pinto et al.[21] found that diagnostic sensitivity of the typical qPCR assay[58] was unaffected when DNA was extracted from pools of up to four skin swabs. While the authors specifically recommend against pooling in trade and quarantine settings given the risks associated with the failure to detect a single infected individual[21], their results suggest pooling could reduce several-fold the number of DNA extractions and qPCR reactions required to achieve the EU's requisite detection probability in a shipment (Figs. 1 and 2). Again, emperical tests of sensitivity in real-world settings, especially with low-level Bsal infections, are needed to evaluate the actual when pooling samples. Also, it is worth remembering that the same number samples must be collected with or without pooling; efficiencies are only gained in the processing and screening steps.

Environmental DNA

Environmental DNA samples are being used to detect pathogens in a growing number of settings, from natural environments[66–69] to ballast water[70] and even in trade[29,62,63]. However, while the statistical framework for making proper inference from eDNA results is well-developed in certain contexts (e.g., the presence or absence of a target species in ponds using occupancy models[68,71]), these approaches do not translate well to small, finite populations. I therefore developed a new formula for such settings (equation (5)).

The formula makes two key assumptions. First, it assumes that the eDNA shed into the water is homogeneously distributed in the water. While pathogen eDNA is likely clumped (e.g., Bsal may be found principally in skin sheds), one could homogenize the water (e.g., with a blender) prior to taking samples to meet this assumption, at least in the smaller volumes used to house animals in shipments or captive populations. Second, as with the formula for pooled samples, it assumes that test sensitivity is unaffected by the number of hosts in the water at the same time. That is, the target pathogen eDNA is not swamped by non-target host and microbial eDNA or other waste material leading to inhibition of the PCR reaction. This assumption needs to be empirically verified, but it may be possible to minimize PCR inhibition with dedicated kits[68] or by diluting the eluted DNA prior to PCR, although such dilutions risk reducing sensitivity. In any case, the formula I present could be extended to account for this swamping effect if needed.

These assumptions have two important consequences for eDNA-based pathogen detection. First, every sample collects eDNA from the *entire* population. Environmental DNA avoids the issue of whether rare infections are included in any random sample of individuals—they are all sampled because the eDNA from all individuals is distributed homogeneously in the water. The question is only whether there is sufficient target eDNA in a sample to ensure a reasonable probability of a positive test. As a consequence, eDNA sampling can detect rare infections with many fewer samples than individual swabs, or pools of swabs, even when diagnostic sensitivity is very low (Figs. 1 and 2). (I consider various factors that are likely to affect sensitivity in the next section.) This also means that the probability of detecting an infection is, all else being equal, independent of population size (Fig. 2). Thus, while individual-level swabbing can be more efficient than eDNA in small populations, eDNA always outperforms swabbing, even when pooled, in larger populations (Fig. 2). Moreover, because individual sampling must first include infected individuals in the sample in order to detect an infection, sample sizes necessarily increase with population size. But samples sizes for eDNA-based detection *decrease* with population size when prevalence is held constant because there are more infected individuals shedding pathogen eDNA (Fig. 1). Once again, it is essential to establish the actual performance of eDNA-based detection in real world contexts and test how it scales with population size and other variables.

Quantitative nature of detection

Diagnostic sensitivity is usually defined in the context of a classification problem with binary infection status determined by a gold standard of infection (e.g., histopathology) or experimental treatments. As such, sensitivity is generally assumed to be fixed, that is all infections within a population or experiment are equally detectable. However, any test that reacts directly with the pathogen (e.g., pathogen DNA or antigens) or host responses (e.g., antibodies) is necessarily dose-dependent (Fig. 3; equation (6)). The variation in the amount of the analyte (e.g., pathogen DNA) between infected individuals may be small enough to be ignored (but see e.g., Rimmer et al.[72]), or the concentrations generally large enough that detection is assured (e.g., with clearly diseased animals), in which case it may be reasonable to assume diagnostic sensitivity is constant. At the other extreme, recently infected individuals, sub-clinical carriers, or otherwise inapparent infections tend to produce or shed little analyte and are thus much less easily detected than clinically infected animals. Quantitative real time PCR of swabs, for instance, often fail to detect low-level Bd infections[73]. Variation in the status or intensity of infections among individuals can make actual diagnostic sensitivity quite variable. Indeed, the variability among the component samples of a pool can have large effects on the pool-wide sensitivity[64]. However, the the concentration-dependent nature of detection will be especially pronounced with eDNA sampling.

The concentration of target DNA in an eDNA sample co-varies with with infection intensity, as with individual samples[23], but also depends on the conditions in which animals are held (e.g., temperature, volume, time in the water; Fig. 3) and details of

the sampling method (volume sampled, pore size, storage conditions[74]). Thus, one would expect a great deal more variability in sensitivity of eDNA between settings and studies, not to mention between taxa, than with traditional sampling directly from animals. Empirical validation of eDNA-based pathogen detection should thus focus on understanding the causes and consequences of this variability across consignment types, holding conditions, and sampling approaches, and work to develop standards. Equations (6)–(9) are an initial attempt to explore these influences. It is worth noting, however, that researchers may have control over certain key variables, such as the duration and volume in which animals are held before collecting samples, allowing them to maximize sensitivity.

Discussion

While efficiency is not the only important criteria for a testing scheme (e.g., the expertise, specialized equipment, and costs involved must also be considered), routine pathogen surveillance and thus a "clean trade" becomes more feasible as the number of samples are reduced. Pooling swabs and sampling eDNA are both, in principle, viable approaches to reducing sample sizes, although to much different extents and with different drawbacks in their use.

Pooling and batch-processing samples collected from individual animals (e.g., swabs) can reduce the number of diagnostic tests (e.g., DNA extractions and PCR reactions) required to detect rare infections several fold. While some n samples must still be collected, pooling them into groups of size k means that only n/k pools need be processed, which may substantially reduce time and costs of surveillance. The critical question for pooling is whether and to what degree sensitivity, the probability of correctly identifying infections if present, declines as samples are pooled. Laurin et al.[64] found that sensitivity generally declined with pooling in aquatic pathogens, but there are few studies that properly evaluate this. Sabino-Pinto et al.[21] found that pools of up to four skin swabs had similar sensitivity for Bsal detection, but specifically recommended against it in the case of trade. Even if sensitivity is reduced by pooling, this may be offset by gains in detection afforded by allowing a larger fraction of the population or consignment to be screened, increasing the chances that a rare infected individual is included in a sample. For instance, even if sensitivity is reduced from a probability of one with individual swabs to 0.75 with a pool of four swabs, many fewer assays need be run on the pools than the individual swabs to attain the same confidence of detecting an infection (Fig. 2). Equation (4) can be used to evaluate this trade-off.

Environmental DNA offers a different trade-off. The samples collect genetic material shed into the water (or environment more broadly), so the material is diluted in a larger volume and may be degraded, all of which means that fairly little target DNA may end up in any given eDNA sample. Thus, one would expect the sensitivity of eDNA samples to be quite low relative to individual-based samples (Fig. 3). However, eDNA samples from the entire population, at least in the context of small captive populations or consignments of animals in water, perhaps with the aid of mechanical homogenization. This allows eDNA to circumvent the problem of including rare infected individuals in sample that is intrinsic to individual-level sampling. As a result, even with low sensitivity, eDNA sampling can be much more efficient at detecting infections in large consignments than even perfectly sensitive individual-based samples, even when pooled. Moreover, sample sizes required to detect a rare infection do not increase with population or consignment size (Fig. 2). Sampling eDNA thus offers the promise of dramatically reducing the amount of sampling required to ensure disease freedom in the live animal trade.

The actual performance of eDNA-based detection likely depends on the nature of the consignment (e.g., volume, time animals have spent in water), collection and processing (e.g., volume filtered, presence and removal of PCR inhibitors), and biological realities (e.g., rates of shedding and degradation). It is important to evaluate these factors empirically so that the results can be properly interpreted. The simple model presented in equations (7) – (9) provides a useful starting point, illustrating how eDNA concentrations, and thus detection probabilities, vary with volume, time, and rates of shedding and degradation (Fig. 3). The volume of water into which animals shed, relative to the volume sampled, is a key parameter, one that it is likely to vary substantially among taxa, life stages, and settings. So, too, is the length of time in which animals spend in the water, shedding. The concentration of eDNA in water is expected to approach an equilibrium over time, but the rate at which it approaches the asymptote depends on rate of degradation and thus water quality, temperature, pH, etc. are likely to be important[74,75]. However, unless rates of degradation are exceedingly rapid, one would expect concentrations of eDNA to accumulate over several days. Indeed, a clever investigator could use these factors to increase the sensitivity of eDNA-based testing, for instance by holding animals in small volumes of clean water for longer periods of time to ensure that target eDNA accumulates to readily detectable levels before collecting eDNA samples.

Perhaps the most fundamental question is whether pathogen eDNA is swamped by non target host or microbial DNA or becomes PCR inhibition increases with population size. If swamping or inhibition are a problem then the number of eDNA samples required to attain a particular detection probability would need to increase with population size and equation (5) adjusted accordingly. I must stress that it is only with a clear understanding of how these manifold conditions influence detection probabilities[74] can eDNA-based detection be used reliably in the variety of settings and types of consignments that comprise the live animal trade.

The goal of developing a "clean trade" may be the most realistic strategy for preventing the movement and introduction

of pathogens, such as Bsal, into new areas and naive hosts. But it is a daunting task given the immense number of animals involved in international and regional trade. The formulae developed here place sample pooling and eDNA in closed populations on a firm theoretical foundation. They suggest that pooling individual-level samples and, especially, collecting eDNA can substantially reduce sample sizes required to ensure rare, but important infections are found. Moreover, sampling at key nodes in the distribution networks of the live animal trade can make surveillance even more tractable[10]. It is my hope that these approaches can help bring clean trade into reach for a greater number of taxa, places, and contexts.

Equations

Individual-level sampling

The probability of obtaining x infected individuals (D^+) in a sample of size n taken without replacement from a population of size N, of which d are infected is described by a hypergeometric distribution[76]:

$$P(D^+ = x) = \frac{\binom{d}{x}\binom{N-d}{n-x}}{\binom{N}{n}}. \tag{1}$$

However, diagnostic tests are rarely perfect. Infected individuals are correctly detected with probability Se (diagnostic sensitivity) and uninfected animals correctly test negative with probability Sp (diagnostic specificity); false negatives and false positives occur with probabilities $1-Se$ and $1-Sp$, respectively. Cameron and Baldock[76] extended the hypergeometric model to include imperfect tests, such that the probability of observing x positive tests (T^+) is:

$$P(T^+ = x) = \sum_{y=0}^{d}\left(\frac{\binom{d}{y}\binom{N-d}{n-y}}{\binom{N}{n}} \times \sum_{j=0}^{\min(x,y)}\left[\binom{y}{j}Se^j(1-Se)^{y-j} \times \binom{n-y}{x-j}(1-Sp)^{x-j}Sp^{n-x-y+j}\right]\right). \tag{2}$$

Pooling individual-level samples

When the n samples are divided into m groups of size $k = n/m$, which are then tested as pools, the probability that x of the m pools contain an infected individual in them ($Pool^+$) is described by the "pooled hypergeometric" of Theobald and Davies[65]:

$$P(Pool^+ = x) = \binom{m}{x}\sum_{i=0}^{x}(-1)^i\binom{x}{i}\frac{\binom{N-d}{(m-x+i)k}}{\binom{N}{(m-x+i)k}}, \tag{3}$$

for $\max[0, m-(N-d)/k] \leq x \leq \min(m,d)$.

This model can be extended to include false negatives and false positives in a manner analogous to Cameron and Baldock[76], where the probability of observing x positive tests (T^+_{pool}) for the m pools is:

$$P(T^+_{pool} = x) = \sum_{y=0}^{\min(m,d)}\left(\binom{m}{y}\sum_{i=0}^{y}(-1)^i\binom{y}{i}\frac{\binom{N-d}{(m-y+i)k}}{\binom{N}{(m-y+i)k}} \times \sum_{j=0}^{\min(x,y)}\left[\binom{y}{j}Se^j(1-Se)^{y-j}\binom{m-y}{x-j}(1-Sp)^{x-j}Sp^{m-x-y+j}\right]\right) \tag{4}$$

It is important to note that this formulation assumes diagnostic sensitivity and specificity are not influenced by the composition of a pool. A pool is equally likely to test positive if one or all of the individuals within it are positive, or if the pool is comprised of few or many samples. This assumption may be questionable for large pools, but may be reasonable with small k.

Environmental DNA

Let us assume that target eDNA is well mixed in the water. We can then use a binomial to describe the distribution of positive eDNA tests (T^+_{eDNA}) because taking one sample does not affect the probability of detection in subsequent samples. Retaining the definition of sensitivity as the probability of detecting one infection if present (i.e., $Se = P(T^+_{eDNA}|d=1)$), we obtain the following expression for the probably of observing x positive samples:

$$P(T^+_{eDNA} = x) = \binom{n}{x}\left[1-(1-Se)^d + (1-Se)^d(1-Sp)\right]^x\left[(1-Se)^d Sp\right]^{n-x}. \tag{5}$$

Note that because each eDNA sample collects material from all d infected individuals in the population, success is defined in the binomial as the probability of a positive eDNA sample $[1-(1-Se)^d]$, ignoring false positives, rather than the probability a sample includes an infected individual, as is the case when using the binomial to describe detection probabilities with individual samples taken with replacement. Multiple infected individuals in a population simply increase the amount of target eDNA to be detected and thus the effective sensitivity of the eDNA test. Specificity is assumed to be constant and both are assumed to be independent of the $N-d$ uninfected animals in the population.

The quantitative nature of detection

Any test that reacts directly with a pathogen (e.g., pathogen DNA or antigens) or host responses (e.g., antibodies) is necessarily dose-dependent. Focusing on DNA-dependent detection methods (e.g., PCR), let us assume that every copy, C, of the target DNA sequence in a sample has a small, fixed probability of causing a positive result in a reaction, ϕ. The probability of a positive test result can then be described by a "single-hit" model[77,78]:

$$P(T^+) = 1 - e^{-\phi C}, \tag{6}$$

which results in a typical dose-response relationship (Fig. 3). (A logistic relationship between $P(T^+)$ and $\log(C)$, which is commonly used in studies of analytic sensitivity, yields similar results, but requires an extra parameter.) If the C copies come from a single infected individual, then this function describes diagnostic sensitivity. The magnitude of C in a sample can therefore have a strong influence on sensitivity. As noted in the main text, one would expect greater variation in C with eDNA sample than with individual-level samples because eDNA is not collected directly from an animal, but from the environment, which can also play a role. A simple model is useful in clarifying the factors that likely determine the number of target copies in an eDNA sample.

In this model, the number of copies of target eDNA in a sample increase as the d infected individuals shed into the water at rate ψ, some portion, α, of which ends up in each eDNA sample, and decrease as eDNA degrades at rate δ:

$$\frac{dC}{dt} = \psi d\alpha - \delta C. \tag{7}$$

The solution is:

$$C(t) = C_0 e^{-\delta t} + \frac{\psi d\alpha}{\delta}\left(1 - e^{-\delta t}\right), \tag{8}$$

where C_0 is the initial concentration in the water (the first term on the right-hand side is zero when $C_0 = 0$). The solution asymptotes to $C^* = \psi d\alpha/\delta$ over time at a rate dependent upon δ. Substituting the equilibrium solution into the single-hit model (equation (6)), one can see that detection probability depends on these rates in a straightforward way:

$$P(T^+_{eDNA}) = 1 - e^{-\phi C^*} = 1 - e^{-\phi \frac{\psi d\alpha}{\delta}}. \tag{9}$$

While this model has several terms, they have clear biological meaning and could be estimated from experimental data[79–81]. Moreover, they enter the model as a product, with equivalent effects, at least at equilibrium. That is, doubling the volume of water collected in an eDNA sample is the same as doubling the number of infected individuals or halving the decay rate. This model also provides a way to link the many factors that can affect eDNA-based detection via their influence on target copy number, C (e.g., degradation, δ, is likely temperature-dependent[81]), or PCR efficiency (e.g., ϕ might be a function of pH and environmental inhibitors[68]).

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Author contributions statement

J.B. conceived, developed, and and wrote the manuscript.

Additional information

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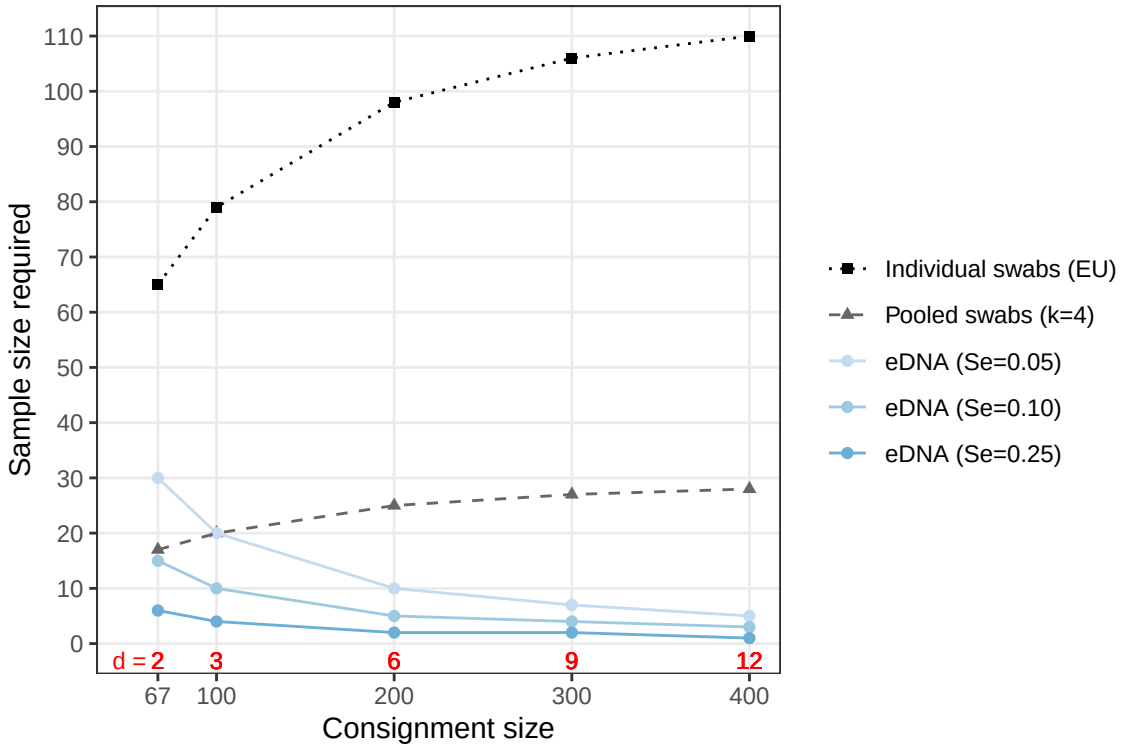

Figure 1. Sample sizes required to achieve a 95% chance of detecting at least one Bsal infection in captive populations or consignments of various sizes using individual swabs (based on EU regulations[59]), pools of four swabs, or eDNA. Calculations are based on a diagnostic sensitivity of 0.8 for swabs and a sensitivity 0.05 – 0.25 for eDNA, and a Bsal prevalence of ~3% in trade[38]. The number of infected animals, *d*, in a consignment given a prevalence of 3% are shown in red at the bottom of the figure. Note that the EU regulations require testing *all* individuals in a consignment of up to 62 individuals[59].

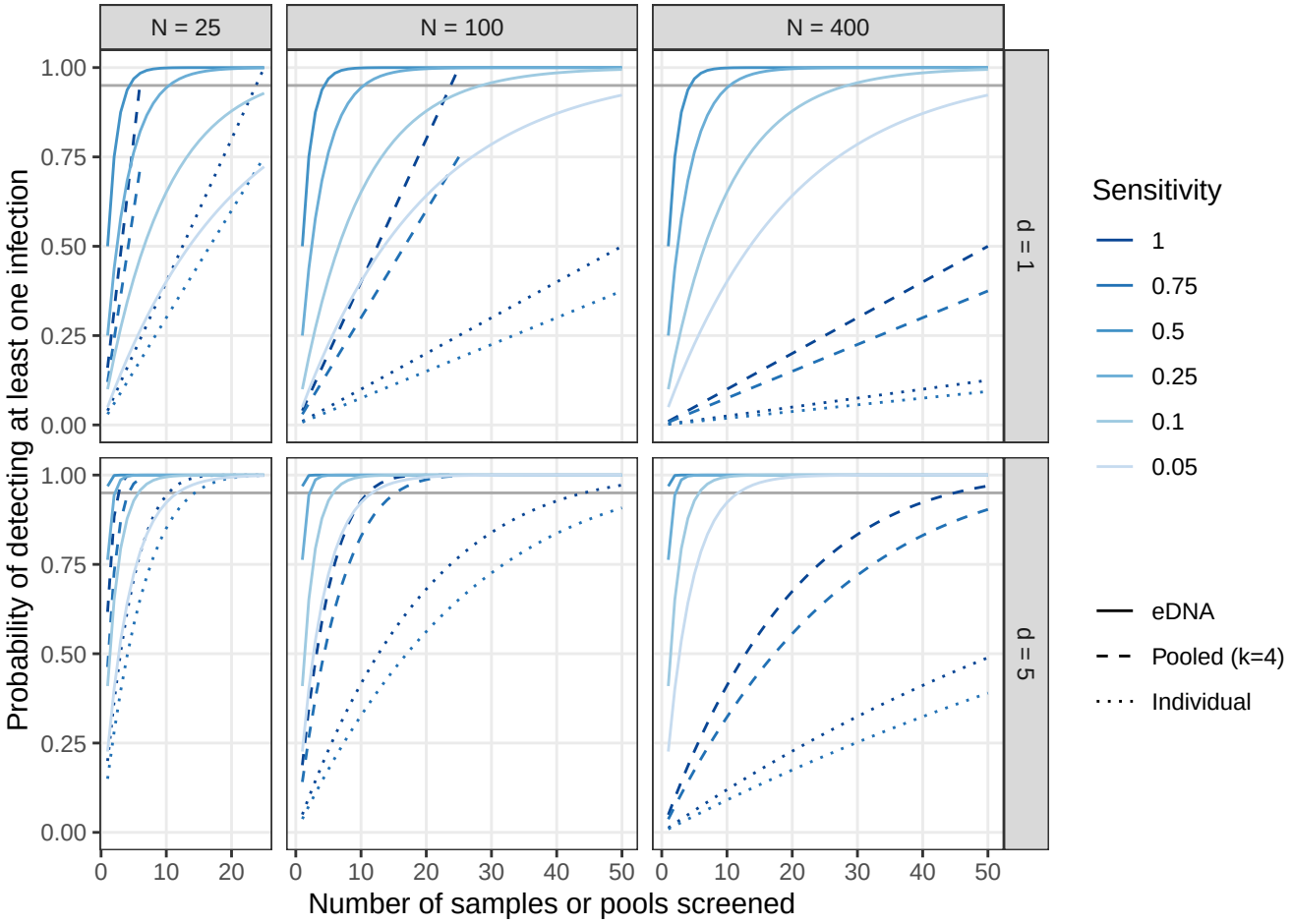

Figure 2. The probability of detecting at least one infection with increasing numbers of samples (or pools) screened, taken without replacement, with $d = 1$ or 5 infected individuals in a population of $N = 25$, 100, or 400. Horizontal gray lines correspond to 95% chance of detection. Note that the maximum sensitivity of eDNA-based tests shown ($Se = 0.5$) is less than the minimum sensitivity presented for individual-based tests ($Se = 0.75$). Specificity is assumed to be 1 in all cases. In contrast with Fig. 1, prevalence of infection declines with population size since the number of infected, rather than prevalence, is held constant.

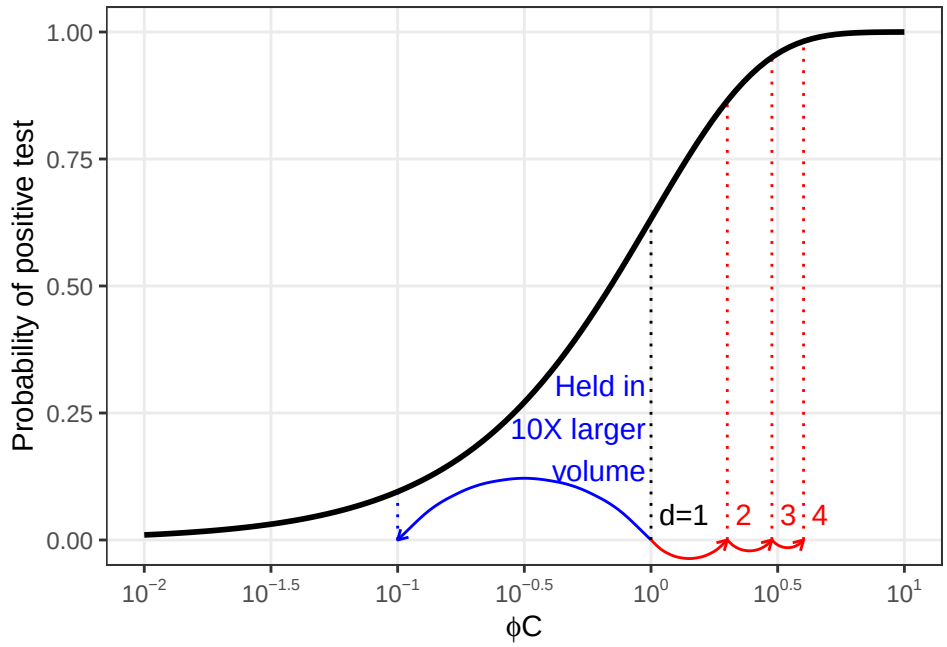

Figure 3. Probability of a positive test result (e.g., with a PCR reaction) as a function (equation (6)) of the per target copy detection rate, ϕ and the number of copies of the target DNA or analyte, C. The arrows and dotted vertical lines show the consequences of increasing the volume of water in which animals are held ten-fold, thus diluting the analyte (blue) or increasing the number of infected animals from one to two, three, or four. Note, however, that the concrete effect of such changes on the probability of a positive test depends on where one starts along the ϕC axis.